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Quenching of Perylene Fluorescence by Co^{2+} Ions in Dipalmitoylphosphatidylcholine (DPPC) Vesicles

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We report the fluorescence quenching of perylene by $CoCl_2 \cdot 6H_2O$ in small unilamellar DPPC vesicles via energy transfer. At the probe-to-lipid ratio of 1:200 and quencher to lipid ratios of $\geq$12.5:1, donor–donor energy transfer between clustered perylene molecules was observed as well as energy transfer from the perylene molecules to cobalt ions both *above* and *below* the main phase transition temperature of the lipid. The fluorescence quenching of perylene by $CoCl_2 \cdot 6H_2O$ in the lipid gel state is shown to be well described by Förster long-range energy transfer when both donor–donor and donor–acceptor energy transfer are considered. In the liquid crystalline phase diffusion of the molecules is described as well as energy transfer. The interaction radius R_0 for energy transfer from perylene to Co^{2+} is found to be $\sim 13.4 \pm 1.1$ Å in the gel phase at 303 K, in good agreement with the theoretical value for R_0 of 13.9 Å. In the liquid crystalline phase at 323 K the lower value obtained for R_0, $\sim 11.3 \pm 1.6$ Å, is attributed to saturation of the Co^{2+} ions at the interfacial region of the bilayer. A diffusion coefficient of $(1.06 \pm 0.15) \times 10^{-6}$ cm^2 s^{-1} is obtained for perylene–cobalt diffusion in the liquid crystalline phase at 323K.

KEY WORDS: Perylene; cobalt(II) ions; lipid bilayers; energy transfer.

INTRODUCTION

Perylene has previously been used as a probe in studies of lipid bilayers and the basic fluorescence and anisotropy properties are well-known [1,2]. As a non-polar, nearly disk-shaped molecule, perylene can easily be integrated into vesicles. Below the phase transition temperature, T_M, diffusion in the membrane is slow, due to its highly ordered state, whereas above the T_M the bilayer is more "fluid" and diffusion can occur.

The fluorescence quenching of perylene by transition metal ions via exciplex formation [3,4] or energy transfer [5,6] has been studied in detail in homogeneous solutions. We have recently reported the long-range energy transfer between perylene molecules and cobalt ions in DPPC liposomes in the gel phase [7]. In this preliminary study we also noted donor–donor energy transfer between the perylene molecules, which we attributed to clustering of the probe molecules in the gel phase of the bilayer. Such donor–donor energy transfer was also noted in fluorescence anisotropy studies of 2,5,8,11-tetra-t-butylperylene [8]. Most fluorescence quenching studies to date in heterogeneous media have been in micellar solutions, where, for example, the quenching of singlet excited pyrene by metal ions [9–12] and the quenching of perylene fluorescence by diazonium salts [13] have been reported.

Theoretical expressions to describe dipole–dipole energy transfer in homogeneous media were first developed by Förster [14] and then by various authors in the regions where Förster's theory breaks down [15–21], Förster's model [14] being strictly valid only for negli-

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gible diffusion at low donor and high acceptor concentrations. The models proposed by Huber [15,16] and Loring, Anderson, and Fayer (LAF) [19] are valid in the region where the donor–acceptor energy transfer is slow in comparison to the donor–donor energy transfer and diffusion is absent. Huber's work is a restricted case of the LAF theory. These models have been verified experimentally in solution by Pandey *et al.* [22] and shown to be still useful in lipid bilayers [7]. When diffusion is occurring, then the models proposed by Gösele *et al.* [20,21] provide a simple and reasonable approximation. Two models are available, depending on the extent to which diffusion is influencing the energy transfer [20,21]. These models have been used experimentally by Tamai *et al.* [23] and Pandey and Pant [24] to describe diffusion-controlled energy transfer between rhodamine 6G and malachite green and between acriflavine and rhodamine 6G, respectively.

In the present paper, we assess the application of various isotropic energy transfer models to anisotropic media in a detailed study of the deactivation of singlet excited perylene by cobalt(II) ions in small unilamellar dipalmitoylphosphatidylcholine (DPPC) vesicles. Previously we have shown that due to the large spectral overlap of perylene emission and cobalt absorption, the fluorescence of perylene is quenched by $[Co(H_2O)_6]^{2+}$ ions via Förster energy transfer in glycerol [6]. Membranes are of fundamental interest in this context because they offer a better-defined separation between donor and acceptor molecules. Understanding fluorescence probe kinetics might then lead to further methods of studying membrane and metal ion interaction. In this case the Co^{2+} ions can be regarded as located at the polar headgroup of DPPC during the short fluorescence lifetime of perylene [25]. We are interested in the two states of the lipid bilayer: (i) the gel state ($L_{\beta'}$ phase), where the effect of diffusion should be small and energy transfer is dominant, and (ii) the liquid crystalline state (L_α phase), where we expect a significant contribution to the quenching from diffusion.

KINETIC MODELS

For the case of resonance energy transfer in the absence of diffusion, Förster gives the fluorescence response function as [14]

$$I(t) = I_o \exp\left(\frac{-t}{\tau_o} - 2\gamma\sqrt{\frac{t}{\tau_o}}\right) \qquad (1)$$

where τ_0 is the lifetime of the unquenched donor and $\gamma = [A]/C_{AO}$ with $[A]$ the acceptor concentration and C_{AO} the critical acceptor concentration for energy transfer. The critical transfer distance R_0 can be expressed by the following equation:

$$R_o^6 = \frac{9\ln(10)\kappa^2\varphi_o}{128\pi^5 N_A n^4}\int_o^\infty \frac{F_d(\tilde{\nu})\varepsilon_a(\tilde{\nu})}{\tilde{\nu}^4}\,d\tilde{\nu} \qquad (2)$$

Here φ_0 is the emission quantum yield of the donor in the absence of the acceptor, κ is the orientation factor, and n is the refractive index of the solvent. $F_d(\tilde{\nu})$ is the emission intensity of the donor and $\epsilon_a(\tilde{\nu})$ is the extinction coefficient of the acceptor at the wavenumber $\tilde{\nu}$. For donor and acceptor molecules which rotate freely and rapidly relative to the donor fluorescence lifetime, $\kappa^2 = 0.67$. This is the case above the phase transition in the lipid but not below, where the perylene motion is restricted [2]. However, when either the donor or the acceptor molecule is free to rotate, as is the case here with the Co^{2+} ions, then the extreme values for κ^2 are 1.33 and 0.33, resulting in a maximum error in R_0 due to assuming $\kappa^2 = 0.67$ of approximately 12% [26,27]. The critical concentration C_{AO} is related to R_0 by

$$C_{AO} = \frac{3}{2\pi^{3/2} N_A R_o^3} \qquad (3)$$

When the rate of energy transfer from donor to donor is comparable to that from donor to acceptor, then the donor fluorescence decay is more appropriately described by a modified form of the Förster equation as proposed by Huber [15,16]:

$$I(t) = I_o \exp\left(-\frac{t}{\tau_o} - (\sqrt{2}\,\gamma_D + 2\gamma_A)\left(\frac{t}{\tau_o}\right)^{1/2}\right) \qquad (4)$$

where the subscripts D and A refer to donor and acceptor, respectively. The reversible migration of energy among the donor molecules is accounted for by the reduced factor for the donor transfer [15].

When the effects of diffusion are also present in the system as well as energy transfer, then the expressions given by Gösele *et al.* [20,21] are more appropriate. There are two forms of the donor decay, depending on the extent to which diffusion is influencing the energy transfer kinetics. They are

$$I(t) = I_o \exp\left(-\frac{t}{\tau_o} - 4\pi D r_F [A] N_A t - 2\gamma\left(\frac{t}{\tau_o}\right)^{1/2}\right) \qquad (5)$$

for $r_F/r_{AD} > 1$

and

$$I(t) = I_o \exp\left(-\frac{t}{\tau_o} - 4\pi D r_{AD}[A]N_A t - 8r_{AD}^2[A]N_A(\pi D)^{1/2} \right) \quad (6)$$

for $r_F/r_{AD} < 1$

where r_{AD} is the collision radius where immediate energy transfer is assumed to occur and r_F is an effective trapping radius given by

$$r_F \approx 0.676 \left(\frac{R_o^6}{\tau_o D} \right)^{1/4} \quad (7)$$

where $D(= D_D + D_A)$ is the sum of the donor and acceptor diffusion coefficients.

In a high-viscosity solvent, where the diffusion length of the donor and acceptor is much shorter than the critical transfer distance, Eq. (5) can be simplified to Förster's model, which is given in Eq. (1). Equation (6) is analogous to the Smoluchowski expression for pure diffusion-controlled reactions where no long-range energy transfer is present.

MATERIALS AND METHODS

Perylene (Aldrich Gold Label) was used as received. $CoCl_2 \cdot 6H_2O$ (Merck p.a.) was recrystallized from methanol. L-α-Dipalmitoylphosphatidylcholine (DPPC) was obtained from the Sigma Chemical Co.

The buffer solution was piperazine-*N,N'*-bis-2-ethane-sulfonic acid (PIPES) (Biochemical BDH) with KCl (Aldrich) and EDTA (Aldrich). NaN_3 (Aldrich) was added to obtain a pH of 7 [28].

Small unilammelar vesicles (SUV) were prepared using methanol injection at 328 K, which is above the phase transition temperature for DPPC (T_M = 314 K). The lipid-to-probe ratio in all cases was 200:1. The concentration of the lipid in the buffer was $6.18 \cdot 10^{-4}$ *M*.

Fluorescence spectra were recorded with a Perkin–Elmer MPF-44 E spectrofluorimeter with a quantum correction unit and thermostated cuvette holder. All fluorescence spectra were corrected for reabsorption of the $[Co(H_2O)_6]^{2+}$ complex, using the method of Marciniak [29].

The quencher concentration was checked spectrophotometrically, where the extinction coefficient of the $[Co(H_2O)_6]^{2+}$ complex was determined to be ϵ_a(515 nm) = 13 l mol^{-1} cm^{-1}. Fluorescence decay kinetics were measured using the technique of time-correlated single-photon counting [30], with lamp and fluorescence data collected simultaneously, to correct for any possible variations in the excitation pulse profile [31]. Hydrogen was used as the filler gas for the lamp. Operating the lamp at 40 kHz, the instrumental pulse width was about 1.5 ns fwhm. The sample temperature was controlled by electrical heating (Eurotherm) with a precision of ±0.5 K. The maximum number of counts in a channel was between 1.10^4 and 2.10^4. Reconvolution analysis of the decays was performed using the software supplied by IBH Ltd. (Glasgow). Goodness-of-fit criteria were the reduced χ^2, the plot of weighted residuals, and the autocorrelation function. The excitation wavelength for all measurements was 385 nm. Perylene emission was detected at 444 nm. All measurements were carried out below and above the main phase transition temperature of DPPC at 303 K ($L_{\beta'}$ phase) and 323 K (L_α phase).

RESULTS AND DISCUSSION

In small unilamellar·DPPC vesicles in the gel phase (T = 303 K) the fluorescence lifetime of perylene is 6.31 ns, with a χ^2 of 1.31, and hence shows small deviations from a single-exponential function. This we attributed to the effects of donor–donor energy transfer due to the clustering of the perylene molecules in the gel phase [7]. The nonrandom distribution of probe molecules was observed in perylene concentration studies of the lipid bilayer [7]. Hence the number of perylene molecules involved in the energy transfer processes is independent of the size of the perylene cluster once the minimum number of molecules is present for the donor–donor energy transfer process to occur [7]. When increasing the temperature to 323 K (i.e., the liquid crystalline phase), the fluorescence decay function can be fitted better to a monoexponential fit (χ^2 = 1.09), with a reduced lifetime of 5.81 ns. In this phase the perylene molecule will have more mobility and hence probe clustering will be reduced [32]. The fluorescence lifetimes measured here are in agreement with those reported in DMPC and DOPC vesicles [1,2].

When $CoCl_2 \cdot 6H_2O$ is added to the liposome suspension, the fluorescence decay curves cannot be fitted to a monoexponential decay law. The deviations (see χ^2 values in Tables I and II) being significantly higher below T_M. The stronger quenching effect above T_M is evident from the faster fluorescence decay. At $[Co^{2+}]$ =

0.1 M, the mean lifetimes calculated from $\tau_e = \int_0^\infty tI(t)dt / \int_0^\infty I(t)dt$ are τ_e (303 K) = 3.80 ns and τ_e (323 K) = 3.29 ns. In the lipid bilayer the cobalt ions are anchored at the lipid headgroup with an ion-lipid binding constant for Co^{2+} and DPPC of 1.7 M^{-1} [25]. Even at the lowest Co^{2+} concentration of 0.01 M cobalt chloride, the Co^{2+}:lipid ratio is 12.5:1. Hence the metal ion concentration at the headgroup is high. The addition of Co^{2+} to the lipid bilayer did not affect the stability of the vesicles, as a single phase transition was still observed.

Below the phase transition temperature (i.e., in the gel phase), diffusional effects are minimal, hence we analyzed the data in terms of Förster kinetics for both a three-dimensional system [Eq. (1)] and a two-dimensional system as

$$I(t) = I_o \exp\left(\frac{-t}{\tau_o} - 2\gamma\left[\frac{t}{\tau_o}\right]^{1/3}\right) \qquad (8)$$

A two-dimensional model may appear at first glance to be more appropriate for the lipid system that we are studying given that the cobalt ions are bound to the lipid headgroup and the perylene molecules are within the interior of the bilayer. However, as can be seen from Table 1, the χ^2 values are consistently better for the three-dimensional (3D) model, indicating that this model is more appropriate. However, the values obtained for γ are similar for both models. Hence we have calculated values for the interaction radius, R_0, from Eqs. (1) and (2), and these are given in Table I. The values obtained for R_0 calculated from Eqs. (8) and (2), i.e., for a two-dimensional (2D) model, are, within the error, the same as those calculated assuming a 3D system. Below T_M the values obtained for R_0 are not constant as expected but are too high at the low acceptor concentrations (i.e., for $[CoCl_2 \cdot 6H_2O] \leq 0.06$ M). The value for R_0 calculated from the overlap of the absorption spectra of $CoCl_2 \cdot 6H_2O$ and the emission spectra of perylene using Eq. (2) is 13.9 Å in lipid bilayers and 12.4 Å in glycerol. The difference between the two values is due to a blue shift in the Co^{2+} ion absorption spectra. The experimental value for R_0 in glycerol solution is 13.4 Å [6]. Also, the plot of γ versus $[Co^{2+}]$ (Fig. 1) gives a value for R_0 of 12.1 ± 1.6 Å from the slope but does not go through the origin as predicted by Eq. (3). This suggests that an additional energy transfer process is occurring at the low acceptor concentrations. This was found to be donor–donor energy transfer between the perylene molecules, as the rate of donor–donor energy transfer is comparable to the rate of donor–acceptor energy transfer [7]. Radiative energy transfer was excluded, as no evidence was found for this in the steady-state spectra. Analyzing the perylene fluorescence decay with no quencher added in terms of Förster kinetics [Eq. (1)] gave a value for γ_D of 0.120 ± 0.006, which is, within the error, consistent with the intercept of the straight-line graph in

Table I. Best-Fit Parameters at 303 K to Various Decay Functions for the Fluorescence Quenching of Perylene at Different Co^{2+} -Ion Concentrations in DPPC Vesicles

	Co^{2+} (M)							
	0	0.011	0.021	0.031	0.041	0.060	0.080	0.100
Monoexponential								
τ (ns)	6.31	5.88	5.40	4.95	4.71	4.44	4.12	3.91
χ^2	1.31	1.62	2.13	3.11	3.75	3.74	4.02	4.70
Förster kinetics								
2D model								
τ_0 (ns)		5.87±0.06	6.13±0.12	5.89±0.15	5.77±0.15	5.32±0.15	5.26±0.15	5.23±0.18
γ		0.156	0.218	0.298	0.342	0.404	0.407	0.478
χ^2		1.76	1.10	1.33	1.40	1.14	1.34	1.47
3D model								
τ_0 (ns)		6.60±0.21	6.45±0.21	6.38±0.24	6.37±0.24	5.95±0.24	5.96±0.27	6.15±0.30
γ		0.138	0.208	0.299	0.352	0.392	0.430	0.529
χ^2		1.25	1.05	1.22	1.13	1.18	1.23	1.30
R_0 (Å)		17.7±0.3	16.4±0.2	16.3±0.3	15.7±0.1	14.3±0.1	13.4±0.1	13.3±0.1
Equation (4)								
γ_A		0.053	0.123	0.214	0.267	0.307	0.345	0.444
R_0 (Å)		12.9±1.0	13.8±0.5	14.6±0.3	14.3±0.3	13.2±0.2	12.4±0.2	12.6±0.2

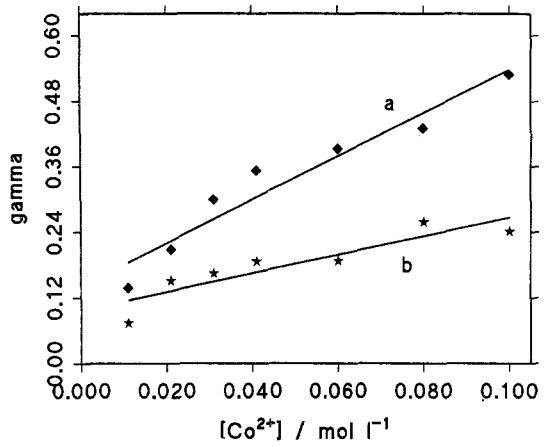

Fig. 1. Plot of γ vs $[Co^{2+}]$ for perylene–cobalt energy transfer in lipid bilayers at (a) 303 K and (b) 323 K.

Fig. 1, i.e., 0.140 ± 0.038. Given this value for γ_D, we can analyze the fluorescence decays using Eq. (4), which takes the effects of donor–donor energy transfer into account. The values obtained for R_0 (see Table I) were then found to be constant, with a mean value of 13.4 Å, in good agreement with the R_0 measured in glycerol and calculated from Eq. (3).

Table II gives the parameters obtained when using Förster's model for both a 2D [Eq. (8)] and 3D [Eq. (1)] system to describe the fluorescence decay data above the phase transition temperature of the lipid. Once again the model of a 3D system is more appropriate, as judged by the χ^2 values, but both models give similar γ and τ_0 values. Here the values obtained for the decrease in τ_0 with increasing Co^{2+} concentration reflect the diffusion of the perylene molecules and cobalt ions as might be expected in the liquid crystalline phase of the lipid bilayer. Below T_M the fluorescence lifetime, τ_0, is almost-constant, showing that little diffusion occurs in the gel phase (see Table I). Figure 2 gives a typical fit to the fluorescence decay with 0.06 M cobalt chloride present at T = 323 K.

We have therefore considered the expressions given by Gösele *et al.* [20,21] for diffusion-controlled energy transfer [Eqs. (5) and (6)] in the liquid crystalline phase. To decide which is the more appropriate expression in our case, we calculated r_F and r_{AD} from our experimental data. For $[Co^{2+}]$ = 0.061 M, we calculated r_F = 8.6 Å, from Eqs. (5) and (7), and r_{AD} = 4.4 Å, from Eq. (6), which gives r_F/r_{AD} = 1.95, indicating that Eq. (5) is more appropriate and energy transfer dominates over diffusion. This is not the case in the solvent ethylene glycol, where $r_F/r_{AD} < 1$, which shows that in this solvent the quenching of perylene fluorescence by cobalt ions is purely diffusion controlled [6].

The parameters obtained from interpreting the fluorescence decay data in the liposome above T_M, using Eq. (5), are given in Table III. The results show evidence of donor–donor energy transfer between the perylene molecules above the phase transition, similar to that observed in the gel phase. When plotting γ versus $[Co^{2+}]$ the straight-line graph does not go through the origin (Fig. 1). R_0 calculated from the gradient of this graph is 9.9 ± 1.8 Å, which is less than, although within the error of, that observed below the phase tran-

Table II. Best-Fit Parameters at 323 K to Various Decay Functions for the Fluorescence Quenching of Perylene at Different Co^{2+} -Ion Concentrations in DPPC Vesicles

	Co^{2+} (*M*)							
Kinetic model	0	0.011	0.01	0.031	0.041	0.060	0.080	0.100
Monoexponential								
τ (ns)	5.81	5.08	4.56	4.36	4.14	3.83	3.60	3.41
χ^2	1.09	1.19	1.65	1.61	1.84	1.69	2.15	1.95
Förster kinetics								
2D model								
τ_0		5.33±0.12	5.00±0.12	4.81±0.12	4.62±0.12	4.26±0.12	4.31±0.13	3.95±0.14
γ		0.084	0.159	0.173	0.192	0.190	0.291	0.258
χ^2		1.06	1.17	1.06	1.20	1.14	1.31	1.19
3D model								
τ_0 (ns)		5.41±0.15	5.18±0.16	4.85±0.18	4.48±0.17	4.47±0.20	4.47±0.20	4.18±0.18
γ		0.074	0.151	0.164	0.186	0.187	0.258	0.241
χ^2		1.05	1.10	1.06	1.19	1.13	1.22	1.15

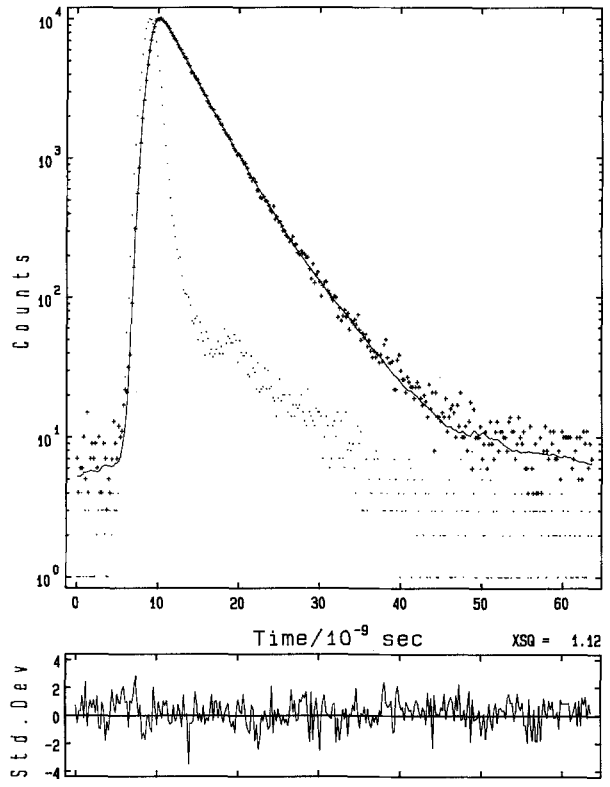

Fig. 2. Reconvolution analysis of the fluorescence decay curve of perylene in DPPC vesicles in the presence of 0.06 M $CoCl_2 \cdot 6H_2O$ at 323 K using the 3D Förster function [Eq. (1)]. The fitted parameters are given in Table II.

sition, where R_0 is calculated to be 12.1 ± 1.6 Å. Taking the data set with no quencher added and analyzing in terms of Förster's model [Eq. (1)] gives $\gamma = 0.055 \pm 0.006$ and $\tau_0 = 6.09$ ns. This γ value is close to the intercept of the graph in Fig. 1 (0.090 ± 0.029), consistent with the presence of donor–donor energy transfer above the phase transition.

Once again, the occurrence of donor–donor energy transfer provides interesting evidence of clustering of the probe molecules. It is notable that this occurs above as well as below the phase transition. A 200:1 lipid-to-probe ratio corresponds to an equivalent molar concentration of $\sim 10^{-2}$ *M*, and yet even at the same concentration in a homogeneous solution of perylene molecules, where self-absorption is the predominant process, radiationless energy transfer between the perylene molecules is not evident, as the fluorescence decays can be fitted well to a monoexponential fit, albeit with an increased lifetime due to self-absorption. However, this may not be surprising given that the mean separation between perylene molecules at this concentration is ~55 Å and the Förster energy transfer radius is 35.7 Å [7]. This provides further supporting evidence for a nonrandom distribution of perylene molecules in the fluid phase of the bilayer. However, the perylene clusters need not be tightly bound for Förster energy transfer to occur. Indeed, the widely reported nonexponentiality in the fluorescence decay for other fluorophores in lipid bilayers, especially in the gel phase, may be caused by interfluorophore interactions such as energy transfer processes due to clustering.

Above the phase transition the diffusion of both the donor and the acceptor molecules as well as energy transfer from donor to donor and also acceptor presents a complex kinetic system. In the treatment which we are using we must emphasize that the evidence points to donor–donor interactions appearing as two terms, namely, Förster energy transfer as if static conditions prevailed and diffusion of the donor sites. In such cases a diffusion term is also needed to describe the donor–donor energy migration; this term not being required below the phase transition. Gochanour *et al.* [18] have derived an approximate expression to account for this behavior at long times and high donor concentrations in terms of an excitation diffusion coefficient, D_E, given by

$$D_E = 0.428 C^{4/3} R_{OD}^2 \tau_0^{-1} \quad (9)$$

Table III. Values Calculated for the Parameters R_0, Both Uncorrected, and Corrected, for Donor–Donor Energy Transfer and *D* from Eq. (5), Which Considers Both Diffusional and Förster Quenching for Perylene Quenched by Co^{2+} -Ions in DPPC Vesicles at 323 K

	Co^{2+} (*M*)						
	0.011	0.021	0.031	0.041	0.060	0.080	0.100
R_0 (Å)							
Uncorrected	14.4±0.5	14.8±0.3	13.3±0.2	12.7±0.2	11.2±0.2	11.3±0.1	10.2±0.1
Corrected	11.2±1.4	13.4±0.6	12.2±0.5	11.7±0.4	10.3±0.3	10.7±0.3	9.7±0.3
D (10^{-6} cm^2 s^{-1})	1.20±0.080	0.92±0.04	1.00±0.03	0.98±0.03	1.31±0.05	0.90±0.02	1.15±0.02

where

$$C=\frac{4}{3}\pi[D]N_A R_{OD}^3 \quad (10)$$

This diffusion coefficient, D_E, forms part of the diffusion coefficient of the donor in Eq. (5), the other part being due to the usual translational diffusion of the perylene and cobalt. We can estimate D_E from the value obtained for $[D]$ of 5.4×10^{-4} M, which comes from the γ value obtained when analyzing the fluorescence decay data with no cobalt ions added. R_0 calculated from Eq. (2) is 35.7 Å. This then gives a value for D_E of 0.22×10^{-6} cm^2 s^{-1}. This is less than the average value obtained for D of 1.06×10^{-6} cm^2 s^{-1} from Table II. The difference between these two values is due to the effects of translational diffusion which will also occur in the fluid phase of the bilayer. Translational diffusion (D_T) can be estimated from Stokes–Einstein theory if the viscosity of the medium is known. Above the phase transition the diffusion length ℓ_{diff} (= 11.4 Å) $\approx R_0$, so if we make the assumption that D_T is similar in the bilayer to that in ethylene glycerol, where ℓ_{diff} is also approximately equal to R_0, then $D_T \approx 0.71 \times 10^{-6}$ cm^2 s^{-1} [6]. The sum of the two diffusion coefficients is then 0.93×10^{-6} cm^2 s^{-1}, which is close to the average diffusion coefficient obtained in Table III.

Table III gives the values obtained for R_0 above T_M when the data are both uncorrected and corrected for donor–donor energy transfer. Once again, the uncorrected data shows a downward trend, while the R_0 values which are evaluated by taking donor–donor energy transfer into consideration are more constant, with an average value of 11.3 Å, which is less than the theoretical value of 13.9 Å. This discrepancy in R_0 values indicates that the local Co^{2+} concentration at the headgroup is lower than the bulk concentration because the interfacial region is saturated with Co^{2+}, consistent with a greater cobalt penetration of the membrane being expected above T_M. Hence the addition of Co^{2+} ions leads to no further quenching of the perylene fluorescence. The values we have obtained for R_0 and D demonstrate that this complex system can be reasonably modeled by a simple isotropic treatment to obtain sensible results.

The percentage of perylene molecules which are transferring energy to the cobalt ions can be estimated from the steady-state spectra. Figure 3a shows plots of the relative fluorescence quantum yield ϕ_0/ϕ of perylene versus quencher concentration. Below and above T_M the plots show a negative departure from linear Stern–Volmer behavior (the classic curvature associated with a fluorophore in environments with different susceptibilities to quenching [33]) rather than the positive curvature associated with Förster energy transfer. In the case of the lipid bilayer there are isolated perylene molecules as well as those associated with the perylene cluster and also the cobalt ions, which will be either bound or close to the polar headgroup of the lipid molecules. Figure 3b shows the straight-line graph obtained when analyzing the data with the following equation, more usually associated with Stern–Volmer quenching [33]:

$$\frac{I_o}{\Delta I} = \frac{1}{f_a K[A]} + \frac{1}{f_a} \quad (11)$$

where $\Delta I = I_0 - I$, f_a is the fraction of the probe

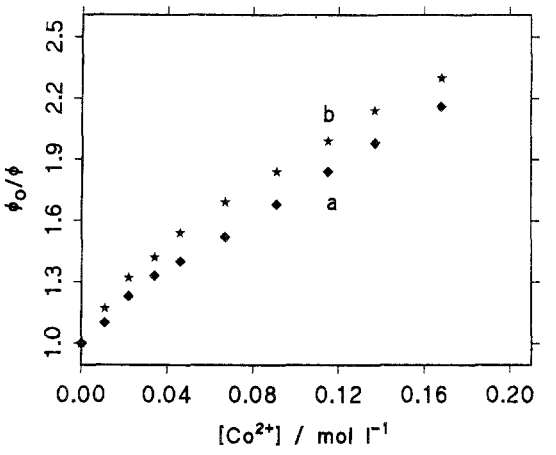

Fig. 3. Stern–Volmer plot for the quenching of the fluorescence of perylene by Co^{2+} ions in DPPC vesicles at (a) 303 K and (b) 323 K.

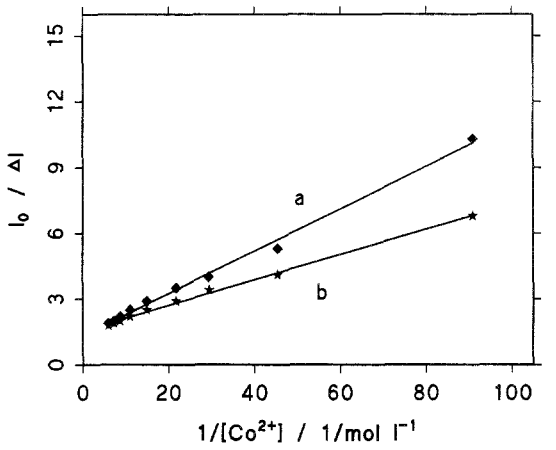

Fig. 3b. Modified Stern–Volmer plot from Eq. (11) at (a) 303 K and (b) 323 K.

molecules accessible to the quencher, and K is a quenching constant (analogous to the Stern–Volmer treatment) associated with this fraction. In our case f_a can be interpreted as the fraction of perylene molecules which transfer energy to the cobalt ion via Förster energy transfer. From Fig. 3b, f_a is 76.0 ± 11.6% for the gel phase and 64.6 ± 4.0% for the liquid crystalline phase. These percentages are, within the error, the same. We can therefore conclude that ~70% of the perylene molecules are transferring energy to the cobalt ion and the remaining 30% are perylene molecules which are either isolated or in clusters and involved in donor–donor energy transfer that is not eventually quenched by the cobalt ion. It is probable that the perylene molecules which are located in the center of the cluster are inaccessible to the cobalt ion and hence more likely to transfer energy to another perylene molecule. Thus the perylene molecule is found in environments which have different susceptibilities to the cobalt ion and the diminishing effect on quenching of increasing the Co^{2+} concentration leads to saturation in the extent of Förster quenching by Co^{2+}, i.e., ϕ_0/ϕ levels off.

CONCLUSIONS

In small unilamellar vesicles, the fluorescence of perylene is readily quenched by Co^{2+} ions via Förster energy transfer. Donor–donor energy transfer between perylene molecules due to clustering effects is shown to occur both above and below the main phase transition of the lipid. Once this effect has been accounted for, the transfer of energy from the perylene molecules to the cobalt ions is shown to follow Förster kinetics below the phase transition temperature and simple diffusion theory above the phase transition. As such, time-resolved fluorescence quenching techniques may prove to be more useful in studying the diffusion of metal ions in membrane systems than the complexity of such systems would at first indicate. Further work has been undertaken to investigate the energy transfer process from perylene molecules to other metal ions and we have recently reported the case of the nickel ion, for which quenching kinetics different from those of the perylene–Co^{2+} system are observed [34].

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